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Published in:
Stem Cell Research

DOI:
[10.1016/j.scr.2018.101368](https://doi.org/10.1016/j.scr.2018.101368)

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Frederiksen, H. R., Holst, B., Ramakrishna, S., Muddashetty, R., Schmid, B., & Freude, K. (2019). Generation of two iPSC lines with either a heterozygous V717I or a heterozygous KM670/671NL mutation in the APP gene. *Stem Cell Research*, 34, [101368]. <https://doi.org/10.1016/j.scr.2018.101368>



Lab Resource: Multiple Cell Lines

Generation of two iPSC lines with either a heterozygous V717I or a heterozygous KM670/671NL mutation in the APP gene

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia, affecting millions of people worldwide. Mutations in the genes *PSEN1*, *PSEN2* or *APP* are known to cause familial forms of AD with an early age of onset. In this study, specific pathogenic mutations in the *APP* gene were introduced into an iPSC line from a healthy individual by the use of CRISPR-Cas9. The study resulted in the generation of two new cell lines, one carrying the V717I *APP* mutation and one with the KM670/671NL *APP* mutation.

Resource table

Unique stem cell lines identifier	1. BIONi010-C-37 2. BIONi010-C-38
Alternative names of stem cell lines	1. BIONi010-C London (V717I) 2. BIONi010-C Swedish (KM670/671NL)
Institution	Bioneer A/S Hørsholm Denmark and University of Copenhagen (UCPH) Copenhagen Denmark
Contact information of distributor	Contact at Bioneer: Benjamin Schmid, bsc@bioneer.dk Contact at UCPH: Kristine Freude, kkf@sund.ku.dk
Type of cell lines	iPSCs
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal plasmids (Okita et al., 2011)
Multiline rationale	Mutated clones
Gene modification	YES
Type of modification	Induced point mutation
Associated disease	Alzheimer's disease (AD)
Gene/locus	1. <i>APP</i> / Chr21:27264096 2. <i>APP</i> / Chr21:27269939 and Chr21:27269938
Method of modification	CRISPR-Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	February 2018
Cell line repository/bank	1. (https://hpscereg.eu/cell-line/BIONi010-C-37) 2. (https://hpscereg.eu/cell-line/BIONi010-C-38)

Ethical approval

The study was approved by the Ethics Committee of the Capital Region of Denmark (H-4-2011-157), and written informed consent was obtained from the participant before enrolment

Resource utility

The exact mechanisms leading to Alzheimer's disease (AD) remain unknown. In order to increase our understanding of disease development and underlying cellular pathological mechanisms, we have established two novel iPSC lines containing either a heterozygous V717I mutation or a heterozygous KM670/671NL mutation in the Amyloid precursor protein (APP) gene. These mutations are known to result in familial forms of AD.

Resource details

Mutations in one of the genes *PSEN1*, *PSEN2* and *APP* are known to result in familial forms of AD. The two mutations V717I (London) and KM670/671NL (Swedish) in the *APP* gene are both used in various mouse models, such as the common 5xFAD (Oakley et al., 2006). Due to their strong phenotype, these specific mutations are highly relevant for establishment of iPSC based disease models that carry a single pathogenic mutation. The iPSC model is supported by other models and patient phenotype information, which will facilitate validation of the *in vitro* model and provide further knowledge about the mutations and AD.

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<https://doi.org/10.1016/j.scr.2018.101368>

Received 2 November 2018; Received in revised form 11 December 2018; Accepted 13 December 2018

Available online 24 December 2018

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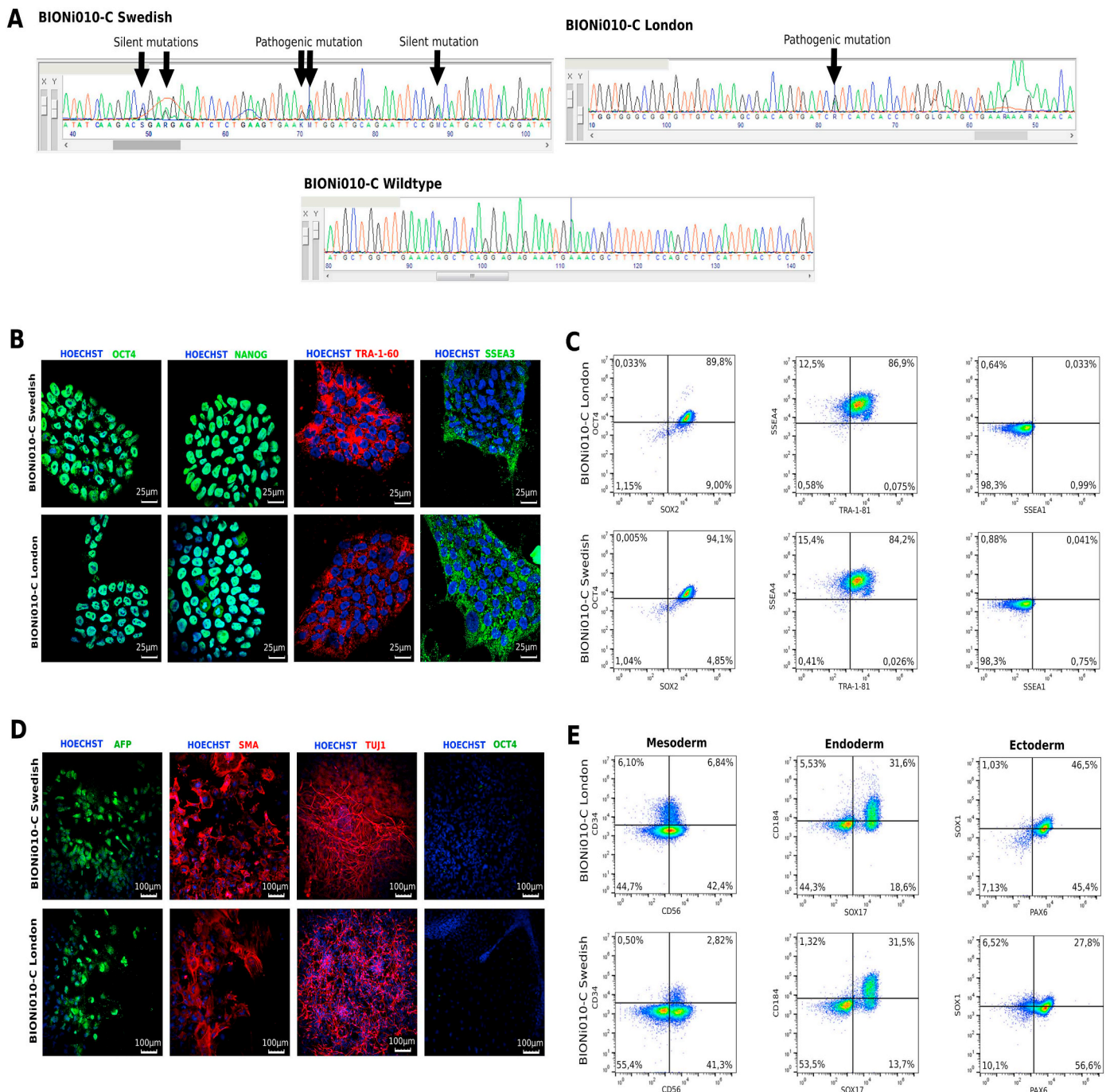


Fig. 1. Characterization of two iPSC lines with either a heterozygous V717I or a heterozygous KM670/671NL mutation in the *APP* gene.

(A) DNA sequencing for the heterozygous V717I (London), heterozygous KM670/671NL (Swedish) *APP* mutation and parental non gene edited iPSC line. (B) ICC for pluripotency markers: OCT4 (green) nuclear labeling, NANOG (green) nuclear labeling, TRA-1-60 (red) cytoplasmic labeling and SSEA3 (green) cytoplasmic labeling. Scale bar represents 25 μ m. (C) FACS analysis of pluripotency markers: OCT4 + SOX2 and TRA-1-81 + SSEA4. SSEA1 served as a negative control for pluripotency. Upper right quadrant in the FACS plots represents double positive cells, thereby determined as pluripotent. (D) ICC for differentiation markers representing the three different germ layers: AFP (green) for endoderm, SMA (red) for mesoderm and TUJ1 (red) for ectoderm. OCT4 labeling served as negative control for successful spontaneous differentiation. (E) FACS analysis of differentiation markers: CD56 + CD34 (mesoderm), SOX17 + CD184 (endoderm) and PAX6 + SOX1 (ectoderm). Upper right quadrant in the FACS plots represents double positive cells, thereby determined as differentiated into the respective lineage.

For this study, the V717I mutation or the KM670/671NL mutation was inserted into the human iPSC line BIONi010-C, which had earlier been established from a skin biopsy obtained from a healthy male individual aged 18 (Rasmussen et al., 2014).

To generate a cell line containing the V717I mutation, the point

mutation resulting in an amino acid change from valine (V) to isoleucine (I) was knocked into the *APP* gene by using the CRISPR-Cas9 system (Jinek et al., 2012). The CRISPR system was also used to insert the KM670/671NL double mutation resulting in an amino acid change from lysine (K) and methionine (M) to asparagine (N) and leucine (L)

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
BIONi010-C-37	BIONi010-C London	Male	18	African	GA	AD
BIONi010-C-38	BIONi010-C Swedish	Male	18	African	GT/AC	AD

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Supplementary Fig. 1 panel B
Phenotype	Qualitative analysis by immunocytochemistry	Both cell lines are positive for OCT4, NANOG, SSEA3 and TRA-1-60	Fig. 1 panel B
	Quantitative analysis by flow cytometry	BIONi010-C APP London positive for: 89,8% double positive for OCT4 and SOX2. 86,9% are double positive for TRA-1-81 and SSEA4. 1.02% are positive for SSEA1(control). BIONi010-C APP Swedish positive for: 94.1% double positive for OCT4 and SOX2. 84.2% double positive for SSEA4 and TRA1-81. 0.79% SSEA1(control).	Fig. 1 panel C
Genotype	Karyotype (G-banding), Resolution 300–400	Both 46XY	Supplementary Fig. 1 panel A
Identity	STR analysis	DNA profiling performed	Available with author
		16 sites tested, all match	
Mutation analysis	Sequencing	Successful integration of heterozygous mutation G > A causing V717I and heterozygous integration of the double mutation GA > TC causing KM670/671NL.	Fig. 1 panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR Mycoplasma Test Kit	Supplementary Fig. 1C
Differentiation potential	Directed differentiation	Specific differentiation markers for all 3 germ layers present in both lines after differentiation	Fig. 1 panel E (control with iPSCs) Supplementary Fig. 1 panel D)
	Spontaneous differentiation	Positive for: AFP, SMA and TUJ1. Negative for OCT4	Fig. 1 panel D

into the *APP* gene.

For both mutations, the nucleotide substitution was confirmed by restriction digest (Supplementary Fig. 1E), followed by DNA sequencing. Sequencing analysis confirmed a successful G > A transition causing the V717I mutation on protein level, and a successful G > T and A > C transition causing the KM670/671NL mutation on protein level (Fig. 1A). The two lines are further referred to as BIONi010-C-London and BIONi010-C-Swedish, respectively.

Pluripotency of the gene-edited lines was confirmed by immunocytochemistry (ICC) (Fig. 1B) and quantified by flow analysis (Fig. 1C) showing expression of the pluripotency markers OCT4, NANOG, SSEA3, SSEA4, TRA-1-81, TRA-1-60 and SOX2. The differentiation potential of the iPSCs was tested via directed- and spontaneous differentiation into *endo*-, *meso*- and *ectodermal* cell types. The test showed that the cells were capable of differentiation into all three germ layers (Fig. 1D and E) and that iPSCs do not express any differentiation markers (Supplementary Fig. 1D). A karyotype analysis was carried out to confirm chromosomal integrity (Supplementary Fig. 1A), and a STR analysis was performed to confirm cell line identity (Available with author). The morphology of the cells was investigated by light microscopy and showed a normal morphology throughout the process of gene editing (Supplementary Fig. 1B). Finally, cells were tested negative for mycoplasma (Supplementary Fig. 1C).

Materials and methods

Gene editing

Cells were grown on Matrigel coated plates in E8 media and incubated at 37 °C and 5% CO₂. Cells were passaged every 4 days using Accutase in a ratio of 1:3. When reaching 80% confluency, cells were detached by using Accutase for 5 min after which cells were nucleofected. Nucleofection was made by using the Lonza nucleofector, P3

solution and the CA167 pulse setting Table 1.

Gene editing was performed using the CRISPR-Cas9 system in combination with a specifically designed ssODN (single stranded oligo deoxynucleotide) as homologous template. The template contained either the London mutation or the Swedish mutation in combination with silent mutations (changing the DNA sequence but not the protein sequence) to avoid repeated cutting by the CRISPR-Cas9. The pathogenic mutation is also a recognition site for a specific restriction enzyme. For London, BclI recognize the mutation and for Swedish, TfiI recognize the mutation. The 20 nt crRNA was designed using the software <http://crispr.mit.edu/>. crRNA, tracrRNA, ssODN, and Cas9 protein were all ordered from Integrated DNA Technologies (IDT) and the CRISPR system assembled *ex vivo*.

Genotyping and restriction enzyme

To screen for positive clones, DNA was extracted using the prepGEM kit (ZyGEM) followed by amplification of the gene by using PCR with the AmpliTaq GOLD DNA polymerase (Thermo Fisher) according to the manufacturer's instructions. Annealing temperature: 60 °C, elongation time 30 s, 40 cycles in a T100 thermocycler (BioRad). The primers SURV APP London FW/RV and SURV APP Swedish (IDT) used for PCR, were designed to cover the mutation at locus Chr21:27269939 + 27,269,938 or the mutation at locus Chr21:27264096. These give rise to a 512 bp product size for Swedish and 694 bp for London. (Table 3). The screening was carried out by making a restriction digest on the PCR product, using TfiI for 1 h at 65 °C (Swedish) or BclI for 1 h at 50 °C (London) (New England Biolabs).

Sequencing

The PCR product from positive clones were sequenced by Sanger

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry				
	Antibody	Dilution	Company	Cat # and RRID
Pluripotency Marker	Goat anti-OCT4	1:200	Santa Cruz Cat: sc-8628	RRID:AB_653551
Pluripotency Marker	Rabbit anti-NANOG	1:50	Peptotech Cat: 500-P236	RRID:AB_1268805
Pluripotency Marker	Rat anti-SSEA3	1:100	Biolegend Cat: 330302	RRID: AB_1236554
Pluripotency Marker	Mouse anti-SSEA4	1:100	Biolegend Cat: 330402	RRID: AB_1089208
Pluripotency Marker	Mouse anti-TRA-1-60	1:200	Biolegend Cat: 330602	RRID: AB_1186144
Pluripotency Marker	Mouse anti-TRA-1-81	1:200	Biolegend Cat: 330702	RRID: AB_1089240
Pluripotency Marker	OCT4 PE	1:25	BD Pharmingen Cat: 560186	RRID:AB_1645331
Pluripotency Marker	SOX2 AF647	1:50	BD Pharmingen Cat: 560294	RRID:AB_1645324
Pluripotency Marker	TRA-1-81 AF647	1:25	BD Pharmingen Cat: 560124	RRID:AB_1645449
Pluripotency Marker	SSEA4 PE	1:25	BD Pharmingen Cat: 560128	RRID:AB_1645533
Pluripotency Marker	SSEA1 PE	1:25	BD Pharmingen Cat: 560142	RRID:AB_1645246
Differentiation Markers	Rabbit anti-Alpha-1-Fetoprotein	1:200	DAKO Cat: A0008	RRID: AB_2650473
Differentiation Markers	Chicken anti-TUJ1	1:500	Raybiotech Cat: 119-15_313	RRID:AB_2753341
Differentiation Markers	Mouse anti-Smooth Muscle Actin	1:200	DAKO Cat: M0851	RRID:AB_2313736
Differentiation Markers	PAX6 Cy5.5	1:50	BD Pharmingen Cat: 562388	RRID:AB_2753343
Differentiation Markers	SOX1 PE	1:50	BD Pharmingen Cat: 561592	RRID:AB_10714631
Differentiation Markers	CD34 PE	1:25	BD Pharmingen Cat: 555822	RRID:AB_396151
Differentiation Markers	CD56 APC	1:25	BD Pharmingen Cat: 555518	RRID:AB_398601
Differentiation Markers	CD184 PE	1:25	BD Pharmingen Cat: 555974	RRID:AB_396267
Differentiation Markers	SOX17 AF647	1:50	BD Pharmingen Cat: 562594	RRID:AB_2737670
Secondary antibodies	Donkey Anti-Mouse IgG Alexa fluor 488	1:200	Life technologies Cat#A21202	RRID: AB_141607
Secondary antibodies	Donkey Anti-Mouse IgG Alexa fluor 647	1:200	Invitrogen Cat# A31571	RRID: AB_162542
Secondary antibodies	Donkey Anti-Rat IgG Alexa fluor 594	1:200	Invitrogen Cat# A21209	RRID: AB_2535795
Secondary antibodies	Donkey Anti-Goat IgG Alexa fluor 488	1:200	Invitrogen Cat# A11055	RRID: AB_2534102
Secondary antibodies	Donkey Anti-Rabbit IgG Alexa fluor 488	1:200	Life technologies Cat# A21206	RRID: AB_2535792

sgRNA, ssODN and primers		
	Target	Forward/Reverse primer (5'-3')
G-RNA V7171	APP	GACAGTGATGCTCATCACCT
G-RNA KM670/671NL	APP	ACATGACTCAGGATATGAAG
ssODN V7171	APP	GTGCAATCAATTGGACTCATGGTGGGGGTTGTTCATAGCAGAGTGATCATCACTTTGGTGTGATGCTGGAAGAAAGACATGACATCCATTCATCAT
ssODN KM670/671NL	APP	CAGGTTCTGGTTTGACAAATATACAGCCGAGAGATCTCTGAAGTGAATCTGGATGCAGAAATTCGCCCATGCACACGATATGAAGTTTCATCATCAAAA
SURV APP London FW/RV (Mutation analysis (PCR) V7171)	APP	TGATCGTGCCCTTTCCAGCTT/ AGAACAACTGTAAACCCAGCA
SURV APP Swedish FW/RV (Mutation analysis (PCR) KM670/671NL)	APP	GGCTAGAAAGAGTTTGGGTAGG/CCACCACACCCAGCTAACTCTTTT
SURV APP London seq (Sequencing V7171)	APP	Rv: AATTCCCACTTGGAAACATGC
SURV APP Swedish seq (Sequencing KM670/671NL)	APP	CACCCATTACAAAGTTTAGC

sequencing using the primers SURV APP London seq and SURV APP Swedish seq (Table 3).

Karyotyping and quality control

Quality control was performed after the lines had been banked and the quality controls are listed in Table 2. The general morphology of the cells was investigated daily by light microscopy. Karyotyping was made at passage 41 for cells carrying the APP London mutation (20 passages after nucleofection), and at passage 39 for cells carrying the APP Swedish mutation (18 passages after nucleofection). When cells were 70–85% confluent, karyotyping was initiated by treating cells with Colcemid (Gibco) for 1.5 h after which cells were detached with Accutase for 5 min. Next, cells were incubated with 0.075 M KCl for 30 min at 37°C, and afterwards fixed by using a mixture consisting of 25% acidic acid and 75% methanol. Fixed cells were cooled overnight at -20 °C and then shipped for GTG-band karyotyping at Anand Diagnostic Laboratory, Bangalore where 20 metaphase spreads were counted. STR analysis was performed by extracting DNA with the DNeasy Blood and Tissue Kit (Qiagen) and subsequently analysing the DNA using the AmpFLSTR Identifier PCR Amplification kit (Applied Biosystem) according to manufacturer's instructions. Microbiological contamination was tested by growing 500 µl of the supernatant in LB medium for 2 days at 37°C. Cells were tested for mycoplasma using the PCR mycoplasma test kit (PromoKine) according to manufacturer's instructions.

Immunocytochemistry

Expression of pluripotency markers, were investigated using a Leica DMRB-fluorescence microscope (Leica Microsystems Wetzlar, Germany). Cells were fixed in 4% PFA for 20 min at RT and labeled according to standard ICC procedures by permeabilising fixed cells with 0,2% Triton X-100 in PBS for 20 min at RT followed by 30 min of blocking with 3% BSA. All antibodies were diluted in 3% BSA in accordance with Table 3. The primary antibodies (OCT4, NANOG, SSEA4, SSEA3, Tra-1-60 and Tra-1-81) were incubated at 4 °C overnight and were visualised with the secondary antibodies Alexa488 or Alexa647 (Life Technologies) diluted 1:200 in BSA. Secondary antibodies were incubated for one hour at RT and in darkness. In all samples, Hoechst bisbenzimidazole 33,258 was used to stain the nuclei.

Trilineage differentiation

IPSCs were cultured for differentiation into mesoderm, ectoderm or endoderm following two different approaches. One method is by making directed differentiation for 5 days. For mesoderm differentiation, cells were cultured for 3 days in mesodermal induction medium (MIM) consisting of StemDiff™ APEL™ medium (STEMCELL Technologies) with 0,1% Pen/Strep (Lonza), 25 ng/ml Activin A (Cell Guidance Systems), 30 ng/ml BMP4 (Peprotech), 50 ng/ml VEGF (Peprotech) and 1,5 µM CHIR99021 (Selleckchem). For the last two days cells were cultured in vascular specification medium (VSM) consisting of StemDiff™ APEL™ medium with 0.1% Pen/strep, 50 ng/ml VEGF and 10 µM SB431542 (Selleckchem). For endoderm differentiation, cells were cultured for 5 days in MCDB131–1 medium (Sigma) containing glucose (Sigma), GlutaMAX (Gibco), Pen/Strep, NaHCO3 (Gibco), BSA (Sigma) and Activin A. For the first 24 h 3 µM CHIR99021

was added to the media. For ectoderm differentiation, cells were cultured in neural induction medium (NIM) consisting of 1:1 DMEM/F12 (Gibco) and Neurobasal medium (Gibco). The NIM contains 1% N2 (Gibco), B27 without Retinoic acid (Gibco), GlutaMAX (Gibco), Pen/Strep (Lonza) and the inhibitors SB431542 (Selleckchem) and LDN193189 (Selleckchem). After five days, expression of the markers, PAX6/SOX1 (ectoderm), CD34/CD54 (mesoderm) and CD184/SOX17 (endoderm) were analysed by Flow cytometry.

The other method, involves growing the iPSC as embryoid bodies. Embryoid bodies were formed by treating 80% confluent iPSCs with Accutase for 1 min and detaching them using a cell scraper. Afterwards cells were transferred to low attachment plates where they were left to grow in E8 media for 7 days with media change every second day. After 7 days, they were plated on Matrigel coated plates, and left to spontaneously differentiate for 14 days in fibroblast media (DMEM + 10% FBS + 0.1% Pen/Strep). Afterwards, ICC was performed for smooth muscle-actin (mesoderm), alpha 1-fetoprotein (endoderm) and beta-3-tubulin (ectoderm) and evaluated using a Leica DMRB-fluorescence microscope.

Flow cytometry

Cells for flow cytometry were detached with Accutase for 5–10 min and 0.2×10^5 cells were fixed in a 0.5 ml solution consisting of 1 part Foxp3 fixation solution and 3 parts permeabilisation solution (R&D systems) and incubated at RT for 30 min. Cells were washed in $1 \times$ permeabilisation buffer (R&D systems) after which antibodies diluted in 100 µl permeabilisation buffer, were added for 45 min at RT.

Cells were washed additionally three times and afterwards run at a calibrated BD Accuri C6 Flow Cytometer. 50.000 cells were analysed with the BD Accuri C6 Flow Cytometer system at high speed in 150 µl buffer. The data was analysed by using the FlowJo_V10 software.

Acknowledgements

This work was supported by funding from Innovation Foundation Denmark (NeuroStem and BrainStem) and Alzheimerforegning Denmark

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2018.101368>.

References

- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* (6096), 337.
- Oakley, H., Cole, S.L., Logan, S., Maus, E., Shao, P., Craft, J., ... Vassar, R., 2006. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J. Neurosci.* 26 (40), 10,129–10,140. <https://doi.org/10.1523/JNEUROSCI.1202-06.2006>.
- Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Yamanaka, S., 2011. A more efficient method to generate integration-free human iPS cells. *Nat. Methods* 8 (5), 409–412. <https://doi.org/10.1038/nmeth.1591>.
- Rasmussen, M.A., Holst, B., Tümer, Z., Johnsen, M.G., Zhou, S., Stummann, T.C., ... 2014. Transient p53 suppression increases reprogramming of human fibroblasts without affecting apoptosis and DNA damage. *Stem Cell Rep.* 3 (3), 404–413. <https://doi.org/10.1016/j.stemcr.2014.07.006>.